3′-Immature tRNA^{Trp} is required for ribosome inactivation by gelonin, a plant RNA *N*-glycosidase

Maurizio BRIGOTTI,* Domenica CARNICELLI,* Paola ALVERGNA,* Alessandra PALLANCA,* Rolando LORENZETTI,† Maurizio DENARO,† Simonetta SPERTI* and Lucio MONTANARO*‡

*Dipartimento di Patologia sperimentale dell'Università di Bologna, Via San Giacomo 14, I-40126 Bologna, and †Lepetit Research Center, Gerenzano VA, Italy

Inactivation of ribosomes by gelonin, a ribosome-inactivating protein with RNA *N*-glycosidase activity on 28 S rRNA, requires macromolecular cofactors present in post-ribosomal supernatants. One of these cofactors has been purified from a rat liver extract and identified as an RNA about 70 nt long which in sequence analysis shows a high level of similarity with mammalian (bovine) tRNA^{Trp}. The pattern of the sequencing gel is consistent with the co-existence in the preparation of two 3′-immature tRNA^{Trp} species, missing only A75, or both A75 and C74. In the presence of ATP, CTP and tRNA nucleotidyltransferase, the gelonin-stimulating RNA is a good acceptor of tryptophan. An oligodeoxynucleotide complementary to positions 55 to 72 of mammalian (bovine) tRNA^{Trp} hybridizes with the gelonin-stimulating RNA as demonstrated by gel mobility shift and ribonuclease H digestion. The oligodeoxynucleotide-directed ribonuclease H treatment also abolishes the gelonin-promoting activity of crude preparations of RNA, giving strong evidence that the only active RNA is a tRNA^{Trp}-like molecule.

INTRODUCTION

Gelonin, from *Gelonium multiflorum*, belongs to the large family of plant ribosome-inactivating proteins (RIPs) with *N*-glycosidase activity which catalytically and irreversibly inactivate eukaryotic ribosomes by releasing a specific adenine from the highly conserved 17-base GAGA loop of 28 S RNA (for review, see [1]). Although a hypothetical antiviral role of RIPs in plants is still a matter of speculation [1–3], RIPs have been used to prepare immunotoxins against cancer cells [1] and an activity against HIV-infected cells has been documented for gelonin and other RIPs [4].

Previous work from our laboratory has shown that depurination of isolated ribosomes by gelonin occurs at a very low rate unless ATP and macromolecular cofactors from a post-ribosomal supernatant are also present [5]. This observation is consistent with the 10000-fold difference in the IC_{50} values reported [6] for the RIP acting on the unfractionated rabbit reticulocyte lysate translating endogenous mRNA (IC_{50} = 0.4 nM) and on the translation of poly(U) in a system reconstituted from isolated ribosomes and elongation factors (IC_{50} between 3 and 12 μM).

One of the cofactors responsible for the up-regulation of gelonin has recently been shown to be an RNA, since treatment of a post-ribosomal supernatant with RNases completely destroys its gelonin-promoting activity [7]. In this paper evidence is presented that the RNA involved is a tRNA, namely tRNA^{Trp}, lacking one or two nucleotides at the CCA 3′-end. Although the conserved CCA terminus of tRNA has a major role in the binding of tRNAs to all ribosomal sites [8,9], chemical probing experiments provide direct evidence for the interaction of immature tRNA with ribosomes [10]. In our experimental conditions, a low-affinity complex between the isolated tRNA^{Trp} and ribosomes was observed.

The knowledge of the cofactors involved in the up-regulation of gelonin may be addressed to increase the therapeutic index of this RIP by its use as a drug of choice against cancer and virus-infected cells having a high level of cofactor activity. Moreover, the identification of regulatory cofactors may help to explain the still controversial biological function of RIPs in plants.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (> 5000 Ci/mmol), [5′-^{32}P]pCp (3000 Ci/mmol), [U-^{14}C]protein hydrolysate (52 mCi/m-atom of C) and [^{3}H]-tryptophan (30 Ci/mmol) were from Amersham. T4 RNA ligase and RNase H (1100 units/ml) were from Pharmacia. Gelonin, polynucleotide kinase, aminoacyl-tRNA synthetase from bovine liver and baker's yeast tRNA were from Sigma. Momordin (from *Momordica charantia*) was a generous gift from Professor F. Stirpe of this Department. Ribosomal RNA (5 S) from *Escherichia coli* MRE 600 was from Boehringer and was labelled with [5′-^{32}P]pCp using T4 RNA ligase [11]. pGEM DNA markers (36–2645 bp) were from Promega and were labelled by exchange of the existing 5′-phosphates with [γ-^{32}P]ATP and polynucleotide kinase [12] followed by removal of excess ATP by gel filtration through a P4 column (0.3 cm × 16 cm, equilibrated with 200 mM ammonium acetate) and precipitation of the end-labelled DNA fragments with ethanol. tRNA nucleotidyltransferase was purified from rabbit reticulocyte lysate up to the DEAE-cellulose step [13]. Most of the chemicals for RNA sequencing were from the DNA sequencing kit of DuPont–NEN (Maxam–Gilbert procedure). Two oligodeoxynucleotide probes were synthesized with an Applied Biosystems model 391 PCR MATE synthesizer and purified by gel filtration through P2 columns [14]: the first, 5′-TGACCCCGACGTGATTCG-3′, is complementary to

Abbreviation used: RIPs, ribosome-inactivating proteins.

‡ To whom correspondence should be addressed.

positions 55 to 72 of mammalian (bovine) $tRNA^{Trp}$ (oligo 1); the second, 5′-CCGGGTTCGATTCCC-3′, reproduces a conserved sequence from positions 48 to 62 of (mouse) $tRNA^{Glu}$ and of (mouse, rat) $tRNA^{Gly}$ (oligo 2).

Source of RNA

RNA was obtained from rat liver post-ribosomal supernatant by Fast Flow Q-Sepharose chromatography, followed by phenol extraction and precipitation with ethanol [7].

Assay of the gelonin-promoting activity of RNA (complementation assay)

This was performed as described previously [6,15]. Briefly, 80 S *Artemia salina* ribosomes (10 pmol in 10 μl) were preincubated in the absence and in the presence of RNA with 5 nM gelonin, a concentration of the RIP that is inactive in the absence of cofactors [5]. After 10 min at 28 °C, 2.5 μl samples were withdrawn and the extent of inactivation of ribosomes was quantified in a 100 μl poly(U) translation system.

Purification of RNA by PAGE

RNA was fractionated on a 40 cm 10% polyacrylamide–4 M urea gel. The gel was sectioned and RNA extracted from the crushed gel pieces by incubation in 0.1% SDS, 10 mM magnesium acetate, 0.5 M ammonium acetate at 25 °C with shaking overnight. Before assay for gelonin-promoting activity, the RNA was precipitated with ethanol, separated from gel impurities by chromatography on a Mono-Q column (HR 5/5) and collected from the 0.8 M NaCl peak [7] by ethanol precipitation. A second electrophoresis of the most active fraction was performed through a 20 cm 20% polyacrylamide–7 M urea gel. RNA fractions were likewise tested for gelonin-promoting activity. The active RNA from the second gel was labelled with [5′-^{32}P]pCp and tested for purity and size on a 40 cm 20% polyacrylamide–8 M urea gel.

Binding of RNA to ribosomes

The active RNA (from the 10% polyacrylamide gel) was labelled with [^{32}P]pCp, electrophoresed on the 20% polyacrylamide–7 M urea gel to remove unbound [^{32}P]pCp, eluted and brought to a specific radioactivity of 10000 c.p.m./pmol with the same unlabelled RNA. The binding assay was performed in the same conditions as the first step of the complementation assay and consisted of 10 μl of 10 mM Tris/HCl (pH 7.4), 100 mM ammonium acetate and 3.5 mM magnesium acetate containing 10 pmol of *A. salina* ribosomes and 1–6 pmol of RNA [in 6 μl of 20 mM Tris/HCl (pH 7.0), 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM benzamidine and 0.1% Triton X-100]. After 10 min at 28 °C the samples were diluted with 5 ml of cold buffer [20 mM Tris/HCl (pH 7.4), 20 mM KCl, 10 mM magnesium acetate] and the ribosome-bound RNA was collected on Millipore filters. The filters were washed three times with 5 ml of buffer and assayed for radioactivity. Blank values were obtained by postponing the addition of [^{32}P]RNA after dilution of the samples with the 5 ml portion of buffer.

Sequencing of RNA

The active RNA from the second gel was labelled either with [5′-^{32}P]pCp at the 3′-end [11] or with [γ-^{32}P]ATP at the 5′-end [16] and electrophoresed for 6 h at 2000 V through a 0.75-mm-thick 20% polyacrylamide–8 M urea gel. The band of the ^{32}P-labelled RNA was cut out and extracted. Chemical sequencing of the labelled RNA was performed using the guanosine and adenosine reactions described by Peattie and Gilbert [17] and the uridine reaction described by Peattie [18]. The cytidine reaction was performed both according to Peattie and Gilbert (lane C_1) and according to Peattie (lane C_2). Samples were run on 0.4-mm-thick, 40-cm-long 20% polyacrylamide/8.3 M urea gels at 2400 V.

Oligodeoxynucleotide-directed gel mobility shift and RNase H digestion

Hybridization of the 3′-end-labelled RNA with the oligodeoxynucleotide probes was tested by a gel-mobility-shift assay [19]. RNase H treatment was performed by adding the enzyme (0.22 units/μl) at the end of the hybridization reaction and allowing a further incubation at 37 °C for 20 min. The samples were run on a non-denaturating gel [19] alongside untreated samples.

RNase H treatment in the absence and in the presence of the oligodeoxynucleotide probes was also performed on the unfractionated RNA from Fast Flow Q-Sepharose and the samples were assayed for gelonin-promoting activity.

RESULTS

Purification of the active RNA by PAGE

In the first semi-denaturing gel (Figure 1a), rat liver RNA from the Fast Flow Q-Sepharose peak yielded several bands (lane 2) spanning, as a whole, a region of slightly greater mobility than that occupied by baker's yeast tRNAs (lane 1). Most of the RNA fractions eluted from the gel showed some gelonin-promoting activity, but the most active was eluted from the region containing the two fastest-migrating bands, which were very faint and well separated from the bulk of slower RNAs. This RNA was used for further purification. The fact that a corresponding region was not present in the lane containing yeast tRNA might explain the previous observation [7] that commercial preparations of baker's yeast tRNA were inactive in complementing the activity of gelonin. Also, the assay for amino acid-accepting activity, performed with aminoacyl-tRNA synthetase and [^{14}C]protein hydrolysate on the rat liver RNA eluted from the gel, was positive for the slower-migrating fractions and completely negative for the fastest-migrating bands most active in gelonin complementation.

In the second denaturating electrophoretic step (Figure 1b) the active RNA eluted from the fastest-migrating bands of the previous gel resolved in one major and a few minor bands. The assay for gelonin-promoting activity of equivalent amounts of RNA eluted from the bands showed that only the most prominent band (indicated by an arrowhead) contained active RNA. The amount of this RNA required for the inactivation of 50% of ribosomes in our assay (10 pmol) was about 20 ng (less than 1 pmol, assuming a molecular mass of about 24 kDa for the 70-nt-long RNA; see below), compared with the value of 110–150 ng observed with different preparations of unfractionated RNA from Fast Flow Q-Sepharose.

Finally, the active RNA from the second gel was labelled with [^{32}P]pCp and run on a 40-cm-long denaturating polyacrylamide gel (Figure 1c, lane 1) alongside the end-labelled 5 S RNA (lane 2) and end-labelled DNA fragments of known size as molecular mass markers. The active RNA gave only one major radioactive band. From its position and that of the 5 S RNA marker relative

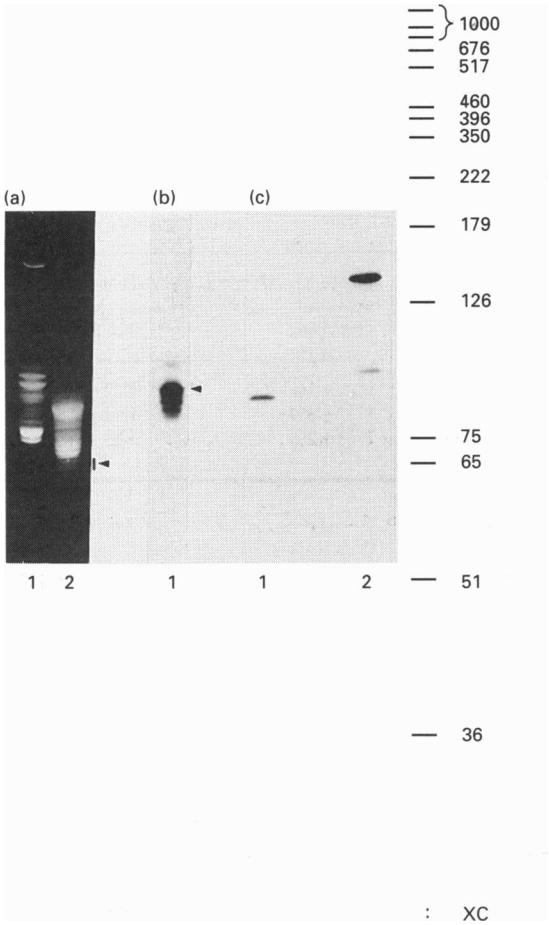

Figure 1 PAGE of RNA

In (**a**), 300 μg of baker's yeast tRNA (lane 1) and 300 μg of the rat liver RNA from Fast Flow Q-Sepharose (lane 2) were heated for 15 min at 55 °C in 50 μl of a solution containing 50% urea, 25 mM NaCl, 4 mM EDTA and 0.02 mg/ml ethidium bromide. After chilling in ice, the samples were diluted with an equal volume of 20% sucrose containing 1 mM EDTA and 0.1% xylene cyanol and loaded on a 10% polyacrylamide–4 M urea gel (1.5 mm thick). Electrophoresis was at 4 °C for 6 h at 600 V. The most mobile RNA was slightly ahead of the blue marker. The preparative lanes from which the rat liver RNA was eluted and tested for complementing activity contained a total of 5 mg of RNA. Ethidium bromide and xylene cyanol were omitted from these samples. In (**b**), the active RNA corresponding to the region indicated by the vertical bar with an arrowhead in (**a**) was labelled with [^{32}P]pCp and resolved on a 20% polyacrylamide–7 M urea gel (0.75 mm thick). Electrophoresis was at 4 °C for 16 h at 400 V. The preparative lane used for eluting and testing RNA was loaded with 70 μg of unlabelled material. In (**c**), the active RNA (lane 1) from the previous step, corresponding to the band indicated by an arrowhead in (**b**), was labelled with [^{32}P]pCp and electrophoresed at room temperature for 6 h at 2000 V on a 20% polyacrylamide–8 M urea gel (0.75 mm thick) together with labelled 5 S RNA (lane 2). Bars and numbers indicate position and size (in bp) of the DNA markers in the same gel; XC, xylene cyanol. Autoradiographies were performed using an intensifying screen.

to the DNA standards, which run faster than RNAs of the same size [20], a length of about 70 nt was calculated.

Binding of the active RNA to ribosomes

A plot of the binding data as $1/b$ against $1/c$, where b and c are the concentrations of bound and free RNA, gave a K_d of about 30 μM. The value was not modified by the presence of gelonin nor by the substitution in the assay of intact ribosomes with ribosomes previously depurinated by momordin, an RIP highly

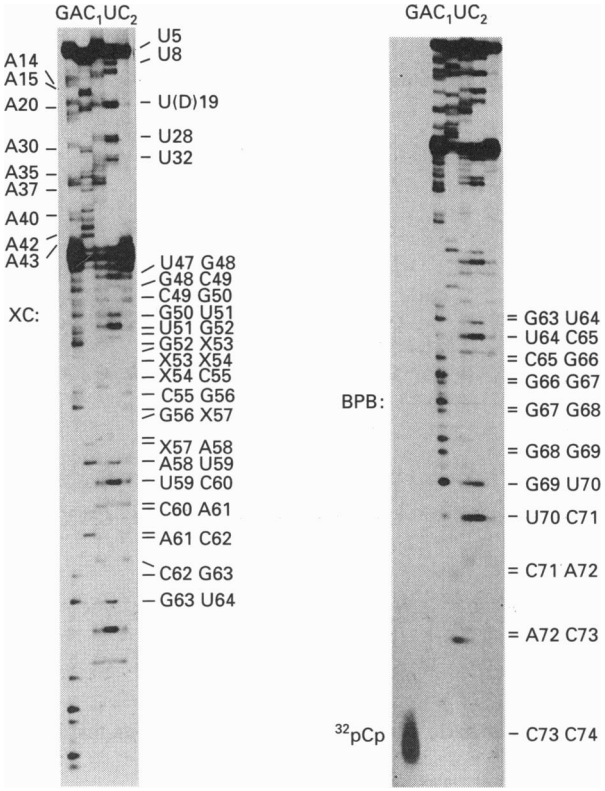

Figure 2 Autoradiograph of the sequencing gel of 3′-end-labelled RNA

Half of the material from each chemical reaction was loaded (left) on a 20% polyacrylamide–8.3 M urea gel and electrophoresed at 2400 V for 90 min, at which time the other half was loaded (right) and electrophoresis continued for a further 90 min. Nucleotides are numbered in the 5′ to 3′ direction. Abbreviations: XC, xylene cyanol; BPB, Bromophenol Blue. For an explanation of the double sequence below the compression zone, see the text. In the upper part of the gel only the positions of the A and U bases are indicated.

efficient as RNA-*N*-glycosidase even in the absence of extra-ribosomal cofactors [6].

Sequence determination

Both the sequencing gel of the 5′-labelled RNA (results not shown) and that of the 3′-labelled RNA (Figure 2) showed a marked compression across nucleotides 44–46, which is typical of several tRNAs [21].

By comparing and combining the readings of the two gels, the sequence reported in Figure 3 was determined. Comparison of the gels was required to ensure identification of the pyrimidine bases, which is doubtful when sequencing RNAs labelled in the 5′-end [22]. In the sequenced RNA, 54 nucleotides out of 74 were identified and 53 (not taking into account post-transcriptional modifications) were identical to those of mammalian (bovine) tRNA^{Trp}, a 75-nt-long tRNA whose sequence is largely conserved in avian and mammalian cells [23–25]. Gaps in the ladders, due to modified bases not detected by the chemical RNA sequencing method, were present in the positions expected from the tRNA^{Trp} sequence ($A_1$57, F54, F53, $F_3$38 and F26; see Figure 3 for definitions). Within the compression zone G_7 appeared in every lane due to the acidic aniline treatment (position 45 in tRNA^{Trp}). The presence of C16 instead of the D16 reported in Figure 3 agrees with the knowledge that in bovine tRNA^{Trp} this position is occupied either by C or by D and in chicken tRNA^{Trp} always

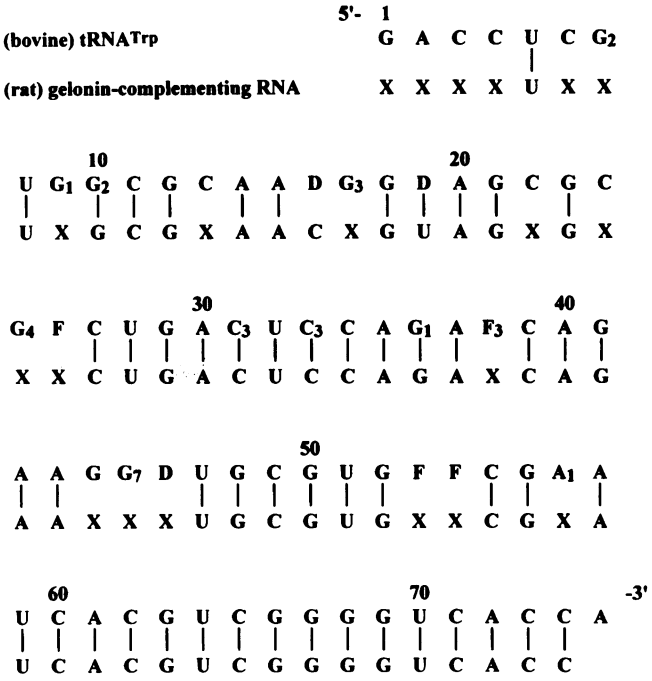

Figure 3 Partial nucleotide sequence of RNA aligned with that of tRNA^{Trp} from bovine liver

X indicates a gap in the ladder or poor definition of a band. F, pseudouridine; F_3, 2′-*O*-methylpseudouridine; A_1, 1-methyladenosine; C_3, 2′-*O*-methylcytidine; D, dihydrouridine; G_1, 1-methylguanosine; G_2, *N*2-methylguanosine; G_3, 2′-*O*-methylguanosine; G_4, *N*2,*N*2-dimethylguanosine; G_7, 7-methylguanosine. Vertical bars indicate identity between corresponding positions without taking into account post-transcriptional modifications.

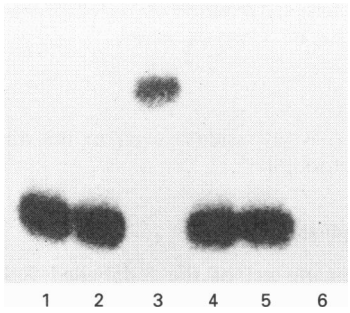

Figure 4 Gel mobility shift and RNase digestion of gelonin-stimulating RNA hybridized with an oligodeoxynucleotide complementary to tRNA^{Trp}

The samples, containing 1 pmol of 3′-end-labelled RNA and 125 pmol of either oligo 1, complementary to tRNA^{Trp}, or oligo 2, not complementary to tRNA^{Trp}, were incubated at 40 °C for 15 min in 8 μl of hybridization buffer [16]. To the samples in lanes 4–6, 2 μl of RNase H was added and the incubation was continued at 37 °C for 20 min. Lane 1, RNA; lane 2, RNA and oligo 2; lane 3, RNA and oligo 1; lane 4, RNA and RNase H; lane 5, RNA, oligo 2 and RNase H; lane 6, RNA, oligo 1 and RNase H.

by C [25]. With these considerations the detected similarity becomes over 80%.

The identification of the RNA as a 3′-immature tRNA^{Trp} molecule came from a careful analysis of the gel shown in Figure 2. Though many steps in the ladder are simultaneously occupied by two bases, or by two bases very close to each other, the sequence can be read assuming the presence of two species, one shortened by one and the other by two nucleotides, with respect to an ideal tRNA with a mature 3′-terminal CCA. With this assumption the nucleotides downstream of the compression zone can be ordered in two sequences identical to bovine liver tRNA^{Trp} missing either A75 alone or both C74 and A75 (see Figure 2).

Table 1 Gelonin-promoting activity of RNA after oligodeoxynucleotide-directed RNase H digestion

Hybridization and RNase H treatment were performed as described in the legend to Figure 4, except that labelled RNA was substituted with 300 ng of unfractionated RNA from Fast Flow Q-Sepharose and 300 pmol of the tRNA^{Trp} complementary oligo 1 was present. The assay for gelonin-promoting activity was performed on the RNA precipitated from the samples with ethanol and resuspended in 3 μl of 20 mM Tris/HCl (pH 7.0), 2 mM EDTA, 0.5 mM EGTA, 2 mM dithiothreitol, 10 mM β-glycerophosphate, 1 mM benzamidine and 0.1% Triton X-100. Values are the means of three experiments performed in duplicate ± S.E.M.

	Poly(U) translation: [^{14}C]phenylalanine incorporated (d.p.m.)	
Treatment	RNA absent	RNA present
None	8605 ± 362	4732 ± 345
RNase H	8737 ± 359	4214 ± 325
Oligo 1 + RNase H	8707 ± 356	8483 ± 359

Gel mobility shift and RNase H digestion

When the 3′-end-labelled RNA was mixed with oligo 1, complementary to positions 55–72 of (bovine) tRNA^{Trp}, all the RNA was retarded (Figure 4, lane 3), indicating that the preparation did not contain RNAs other than species similar in sequence to tRNA^{Trp}. Treatment with RNase H led to complete digestion of the hybridized RNA (lane 6). Oligo 2, which is not complementary to tRNA^{Trp}, neither shifted the mobility of RNA (lane 2) nor induced RNase H digestion (lane 5).

Loss of gelonin-promoting activity of RNA by RNase H digestion

As shown in Table 1, treatment with RNase H in the presence of oligo 1 completely abolished the gelonin-promoting activity of 300 ng of unfractionated RNA from Fast Flow Q-Sepharose, giving strong evidence that the only active RNA in this preparation is a tRNA^{Trp}-like molecule.

Amino acid-accepting activity of the purified RNA

In the absence of tRNA nucleotidyltransferase, the assay for tryptophan-accepting activity performed on purified RNA active

Table 2 Tryptophan-accepting activity of purified gelonin-complementing tRNA

The reaction mixture (25 μl) contained 100 mM Tris/HCl (pH 7.5), 10 mM magnesium acetate, 10 mM ATP, 2 mM CTP, 1 pmol of tRNA, 24 μM [^{3}H]tryptophan (6600 d.p.m./pmol) and RNA nucleotidyltransferase (70 μg as protein) where indicated. After 5 min at 37 °C, aminoacyl-tRNA synthetase (40 units, 10 μg as protein) was added and the acid-precipitable radioactivity was measured after further incubation for 30 min at 37 °C. Values are the means of three experiments performed in duplicate ± S.E.M.

tRNA nucleotidyltransferase	[^{3}H]Tryptophanyl-tRNA formed (pmol)
Absent	0.00 ± 0.01
Present	0.62 ± 0.02

in gelonin complementation was completely negative (Table 2). This result is consistent both with the previous indirect evidence that the active RNA in the Fast Flow Q-Sepharose peak is not a tRNA with an intact CCA tail [7] and with the present sequence data which indicate an immature 3′-end. When, however, the tryptophan-accepting activity was assayed in the presence of ATP, CTP and tRNA nucleotidyltransferase, more than 0.6 mol of tryptophan was accepted per mol of purified RNA. Considering that two or three successive reactions with different yields are required for aminoacylation, this value gives good evidence that the RNA active in gelonin complementation is indeed a 3′-immature tRNA^{Trp}.

DISCUSSION

The requirement of macromolecular cofactors, ATP and tRNA for an efficient inhibition of translation by the RIP tritin (from *Triticum aestivum*) was first reported many years ago [26,27], but these observations were submerged by the dogma that all RIPs act, like ricin A chain, in the absence of cofactors. A renewed interest in the role of cofactors came from the observation that, besides tritin, other RIPs require components of a post-ribosomal supernatant for an efficient inactivation of ribosomes [6] and from the hypothesis that a particular configuration of ribosomes is required for the GAGA loop to be modified by the RNA *N*-glycosidase activity of RIPs [28].

In the present paper conclusive evidence (coming from sequence analysis, amino acid-accepting activity and hybridization with a specific probe) indicates that, in the case of gelonin, an essential cofactor in the post-ribosomal supernatant is a tRNA^{Trp} lacking one or two nucleotides at the 3′ end.

We previously reported that the gelonin-promoting activity of whole post-ribosomal supernatant is much higher than that of the RNA from Fast Flow Q-Sepharose [7], and that this activity requires, besides ATP or GTP [5], the presence of some protein since it is destroyed by proteinase K [7].

The simultaneous requirement of a specific tRNA, a nucleotide triphosphate and protein for the full expression of the gelonin-promoting activity of post-ribosomal supernatants is an intriguing problem. Keeping in mind that the sensitization of ribosomes to gelonin is readily reversible upon removal of macromolecular cofactors and ATP [15], which excludes an enzymically induced modification of ribosomes, and that, as reported in the present paper, the purified tRNA^{Trp}-like molecule by itself binds only poorly to ribosomes, it may be hypothesized that a more stable interaction of the tRNA with ribosomes occurs, in an energy-requiring process, through some protein present in the post-ribosomal supernatant. Alternatively, the sensitivity to proteinase K of the whole supernatant may indicate that the fully active cofactor is a ribonucleoprotein complex which dissociates during fractionation.

Although not yet understood, a role of a tRNA in complementing the activity of an RIP is not inconceivable in view of the growing evidence that sRNA (small RNA), tRNA and tRNA-like molecules have a function in many biological systems. In fact these molecules participate in the splicing of pre-mRNAs [29], in targeting of *de novo* synthesized proteins to the microsomal membrane [30], and in endoribonuclease and prosome activities. The major RNA in prosomes of HeLa cells and duck erythroblasts is tRNA^{Lys-3} [31], and tRNAs lacking their 3′ terminus (tRNA^{Gln}, tRNA^{Gly}, tRNA^{Met} or tRNA^{Arg}) are involved in spermidine-dependent endoribonuclease activities [21]. Certain tRNAs, among them tRNA^{Trp} [32], also act as primers for reverse transcription of retrovirus genomic RNA.

At the moment we cannot envisage a possible mechanism for the action of tRNA^{Trp} in complementing gelonin in ribosome inactivation. Light may be cast on the mechanism by surveying the requirement for specific tRNAs of different RIPs. Work in progress indicates that different RIPs are complemented by different tRNAs.

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